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APPENDIX A

Attached is an article entitled, "Enhanced Immune Reconstitution by Sex Steroid Ablation following Allogeneic Hematopoietic Stem Cell Transplantation," *J. Immunol.* 178:7473-7484 (2007).

Enhanced Immune Reconstitution by Sex Steroid Ablation following Allogeneic Hemopoietic Stem Cell Transplantation¹

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Delayed immune reconstitution in adult recipients of allogeneic hemopoietic stem cell transplantations (HSCT) is related to age-induced thymic atrophy. Overcoming this paucity of T cell function is a major goal of clinical research but in the context of allogeneic transplants, any strategy must not exacerbate graft-vs-host disease (GVHD) yet ideally retain graft-vs-tumor (GVT) effects. We have shown sex steroid ablation reverses thymic atrophy and enhances T cell recovery in aged animals and in congenic bone marrow (BM) transplant but the latter does not have the complications of allogeneic T cell reactivity. We have examined whether sex steroid ablation promoted hemopoietic and T cell recovery following allogeneic HSCT and whether this benefit was negated by enhanced GVHD. BM and thymic cell numbers were significantly increased at 14 and 28 days after HSCT in castrated mice compared with sham-castrated controls. In the thymus, the numbers of donor-derived thymocytes and dendritic cells were significantly increased after HSCT and castration; donor-derived BM precursors and developing B cells were also significantly increased. Importantly, despite restoring T cell function, sex steroid inhibition did not exacerbate the development of GVHD or ameliorate GVT activity. Finally, IL-7 treatment in combination with castration had an additive effect on thymic cellularity following HSCT. These results indicate that sex steroid ablation can profoundly enhance thymic and hemopoietic recovery following allogeneic HSCT without increasing GVHD and maintaining GVT. *The Journal of Immunology*, 2007, 178: 7473–7484.

Allogeneic hemopoietic stem cell (HSC)* transplantation (HSCT) is a potentially curative therapy for a variety of hemological malignancies. Despite improvements in the overall survival of transplant recipients, infections (particularly viral and fungal) remain a major cause of posttransplant morbidity and mortality. In adults, the increased incidence of infections is directly related to prolonged immune deficiency. In contrast, children generally recover immune capacity within ~4–6 mo after HSCT (1). The delay in lymphoid recovery in adult recipients is

dependent on a variety of factors but seems to be primarily related to the age-associated progressive decline of naive T cell export from the thymus (1).

This decrease in T cell output results in a narrowing of the TCR repertoire and a loss of humoral and cell-mediated immunity in adults (2). This thymic involution becomes particularly pronounced after puberty, coinciding with an increase in the production of sex steroids (3–7).

Because thymocyte export is directly proportional to the cellularity of the thymus (8, 9), age-related thymic atrophy results in a gradual decrease in recent thymic emigrants (RTEs) (10, 11) and a decrease in the naive to memory T cell ratio (12–14) resulting in a restricted TCR repertoire in both CD4⁺ and CD8⁺ T cells (15, 16).

In addition, T cell proliferation in response to nonspecific and receptor-mediated (CD3/TCR) stimulation is severely compromised with age (17–19). B cell function is also diminished with age, which is in part, due to the decline in T cell production and subsequent lack of T cell help. However, there are also significant age-associated changes inherent to B cell function (20). Despite B cell numbers remaining relatively constant throughout life due to tightly regulated homeostatic mechanisms, there is a decrease in export from the bone marrow (BM) and a subsequent clonal expansion of peripheral B cells and thus a narrowing of the Ab repertoire (21).

Decreased Ab responses to foreign Ags in the aged are thought to be primarily due to a decline in T cell help (20, 22). However, defective class switching (23) and a preferential loss of high-affinity Abs may play a role (24).

Collectively, these data strongly align aging to a decline in immune capacity involving both cellular and humoral responsiveness. Although these age-related changes appear to be largely benign in healthy individuals, they have a profound impact in

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*Abbreviations used in this paper: HSC, hemopoietic stem cell; HSCT, HSC transplantation; RTE, recent thymic emigrant; BM, bone marrow; ca, castrated; GVHD, graft-versus-host disease; GVT, graft vs tumor; DTH, delayed-type hypersensitivity; Tg, transgenic; TCD, T cell depleted; DC, dendritic cell; TN, triple negative; DP, double positive; SP, single positive; HPRT, hypoxanthine phosphoribosyltransferase; LHRH, luteinizing hormone-releasing hormone.

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severely immunodepressed states, such as HIV infection and therapeutic myeloablation and lymphoablation. In such cases, lymphocyte recovery is severely retarded with age. The atrophic thymus is unable to reconstitute CD4⁺ T cells that are lost during HIV infection (25) and CD4⁺ T cells take three to four times longer to return to normal levels following chemotherapy in postpubertal patients (26).

We and others have demonstrated that surgical and chemical castration both delay the onset of, and reverse, age-related thymic atrophy (3–7, 27, 28). Increases in thymic cellularity, T cell emigration, and peripheral T cell function have been found following sex steroid ablation in aged mice (27) (29, 30). Castration of aged mice also results in an increase in IL-7-responsive B cell progenitors (including late pro-B cells, pre-B cells, and immature B cells) and peripheral B cells (31). This increase in circulating B cells is largely due to an increase in the number of recent BM emigrants (CD45R^{low}CD24^{high}) and these cells remain at an elevated level for up to 54 days after castration (31).

More recently, we have investigated whether the inhibition of sex steroids can be used to enhance the recovery of the hemopoietic system following HSCT, using congenic transplants as a model for autologous HSCT. We showed that sex steroid ablation enhanced thymic reconstitution (27). However, this model does not have the clinical complications of allogeneic HSCT, where the recipients need to balance the polarized effects of GVHD and GVT as well as the increased susceptibility to posttransplant infections. These problems coupled with the regular posttransplant immunodeficiency represent major challenges in the clinic. In this initial study, we also showed preliminary data that chemical castration, using an luteinizing hormone-releasing hormone agonist, increased overall thymocyte number in allogeneic HSCT. However, we did not examine the nature or extent of the T cell or BM recovery and, most importantly, the issues critical to allogeneic HSCT: whether the impact of renewed T cell function, which could also include donor-derived T cells, exacerbated graft-vs-host disease (GVHD) yet retained graft vs tumor (GVT). Therefore, the present study addressed in detail the impact of sex steroid ablation on immune and hemopoietic reconstitution and the levels of GVHD and GVT in the context of allo-HSCT as well as *in vitro* and *in vivo* T cell function.

We found a remarkable increase in T and B cell reconstitution without an exacerbation of GVHD or loss of GVT activity in mice castrated (cx) before HSCT. Proliferation, cytotoxicity, and delayed-type hypersensitivity (DTH) assays were used to determine the function of the lymphocytes produced. IL-7^{-/-} and KGF^{-/-} mice and RT-PCR for several growth factors were used as a means to elucidate the mechanisms by which this enhanced reconstitution occurs.

Materials and Methods

Reagents

Anti-murine CD16/CD32 FcR block (2.4G2) and all of the following fluorochrome-labeled Abs against murine Ags were obtained from BD Pharmingen: Ly-9.1 (30C7), CD127 (IL-7R) (A7R34), TER119 (TER-119) CD3 (145-2C11), CD4 (RM4-5), CD8 β , 2 (53-5.8), TCR- β (H57-597), CD45R/B220 (RA3-6B2), CD43 (S7), IgM-FITC (R6-60.2), CD11b (M1/70), Ly-6G (Gr-1) (RB6-8C5), c-kit (2B8), Sca-1 (D7), CD11c (HL3), I-A^b (11-5.2); isotype controls: rat IgG2a-k (R35-95), rat IgG2a-l (B39-4), rat IgG2b-(A95-1), rat IgG1-k (R3-34), hamster IgG-group 1-k (A19-3), hamster IgG group 2-l (Ha4/8), and 2.4G2 anti-FcR (FcR blocking). Streptavidin-FITC, PerCP-PE also were obtained from BD Pharmingen.

Recombinant human IL-7 was provided by Dr. M. Morre (Cyberis, Yverles, France). It has been confirmed that the proliferative effect of human recombinant IL-7 is equal to murine IL-7 (32). Tissue-culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS,

100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (as well as 50 mM 2-ME for the culture of cells and proliferation assays).

Mice and HSCT

Male C57BL/6J (B6, H-2^b), C3H/HeB6F1/J (B6 \times C3H/F₁; H-2^{b/h}), B10.BR (H-2^b), B6D2F1/J (H-2^{b/h}), CBA/J (H-2^k), BALB/c (H-2^d) B6;129-Fgfr1^{tm1Efu} (KGF^{-/-}) mice were obtained from The Jackson Laboratory and used in experiments when they were between 8 and 12 wk of age. IL-7^{-/-} mice (BALB/c) were provided by Dr. B. Rich (Harvard Institute of Medicine, Boston, MA). KGF^{-/-} and IL-7^{-/-} mice were used between 4 and 7 mo of age. RAG2p-GFP transgenic (Tg) mice (FVB background; H2^b) were provided by M. Nussenzweig (Rockefeller University, New York, NY). HSCT protocols were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. The BM cells were removed aseptically from femurs and tibias. Donor BM was depleted of T cells by incubation with anti-Thy-1.2 for 40 min at 4°C followed by incubation with Low-TOX-M rabbit complement (Cedarlane Laboratories) for 40 min at 37°C. Splenic T cells (for GVHD experiments) were obtained by purification over a nylon wool column. Cells (5×10^6 BM cells with or without splenic T cells and tumor cells) were resuspended in DMEM (Invitrogen Life Technologies) and transplanted by tail vein infusion (0.25-ml total volume) into lethally irradiated recipients on day 0. Before transplantation, on day 0, recipients received 1300 cGy total body irradiation (¹³⁷Cs source) as split dose with 3 h between doses (to reduce gastrointestinal toxicity). Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hypochlorinated drinking water (pH 3.0). Cell lines A20 and P815 were obtained from American Type Culture Collection.

Surgical castration

Mice were anesthetized and a small scrotal incision was made to reveal the testes. These were sutured and removed along with surrounding fatty tissue. The wound was closed using surgical staples. Sham castration required the same surgical procedure, except for the removal of the testes. Castration was performed 1 day before BM transplant for both immune reconstitution and GVHD/GVT studies.

Flow cytometric analysis

BM cells, splenocytes, or thymocytes were washed in FACS buffer (PBS/2% BSA/0.1% azide) and $1-2 \times 10^6$ cells were incubated for 30 min at 4°C with CD16/CD32 FcR block. Cells were then incubated for 30 min at 4°C with primary Abs and washed twice with FACS buffer. Where necessary, cells were incubated with conjugated streptavidin for a further 30 min at 4°C. The stained cells were resuspended in FACS buffer and analyzed on a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software.

Proliferation assays

For one-way MLRs, splenocytes for transplanted mice (4×10^5 cells/well) were incubated for 5 days with irradiated (2000 cGy) BALB/c splenocytes as stimulators (2×10^5 cells/well) in 96-well plates. For pan T cell activation, splenocytes (4×10^5 cells/well) were stimulated with anti-CD3 (145-2C11) and anti-CD28 (37.51) (2.5 μ g/ml as a final concentration of each) for 4 days. In both assays, cultures were pulsed during the final 18 h with 1 μ Ci/well [³H]thymidine and DNA was harvested on a Top Count Harvester (Packard Biosciences). Stimulation indices were calculated as the ratio of stimulated cells (cpm) over unstimulated cells (cpm).

⁵¹Cr release assays

Target cells were labeled with 100 μ Ci ⁵¹Cr at 2×10^6 cells/ml for 2 h at 37°C and 5% CO₂. After three washes, labeled targets were plated at 2.5×10^5 cells/well in U-bottom plates (Costar). Splenocytes cultured with irradiated BALB/c splenocytes (1:2 ratio) for 5 days were added at various E:T ratios in a final volume of 200 μ l to 4–6 wells and incubated for 4 h at 37°C and 5% CO₂. Subsequently, 35 μ l of supernatant was removed from each well and counted in a gamma counter (Packard Biosciences) to determine experimental release. Spontaneous release was obtained from wells receiving target cells and medium only; total release was obtained from wells receiving 5% Triton X-100. Percent cytotoxicity was calculated by the following formula: percent toxicity = $100 \times ((\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release}))$.

Detection of alloreactive T cell clones with intracellular IFN- γ staining

Briefly, splenocytes were incubated for 12–15 h (for secondary allogeneic stimulation with T cell-depleted (TCD), irradiated stimulator cells) with

Brefeldin A (10 μ g/ml), harvested, washed, stained with primary (surface) fluorochrome (FITC, PerCP, and allophycocyanin)-conjugated Abs, fixed, and permeabilized with the Cytofix/Cytoperm Kit (BD Pharmingen), and subsequently stained with anti-IFN- γ PE. FACS analysis was conducted by gating for the designated populations. Flow cytometer and software were used as mentioned below.

DTH assay

Sham-cx and cx mice were sensitized day 42 after allogeneic HSCT by tail vein injection with 200 μ l of 0.01% sheep RBC (Colorado Serum) in PBS. Sensitized animals were challenged at day 46 in the right hind footpad with 50 μ l of 20% sheep RBC suspension while the left hind footpad received the same volume of PBS solution as a control. Forty-eight hours later, footpad swelling was measured with a dial-thickness gauge (Mitutoyo). The magnitude of the response was determined by subtracting measurements of PBS-injected left footpads from the experimental right ones.

Assessment of GVHD

Two models of GVHD were used: C57BL/6J (H-2^b) into C3H/HeJ (H-2^k)—a major mismatch model and B10.BR (H-2^b) into CBA/J (H-2^k)—a minor mismatch model. Three different T cell doses were used in the minor mismatch model: 0.1×10^6 , 0.5×10^6 , and 1×10^6 . The severity of GVHD was assessed with a clinical GVHD scoring system as first described by Cooke et al. (33). Briefly, ear-tagged animals in coded cages were individually scored every week for five clinical parameters on a scale from 0 to 2: weight loss, posture, activity, fur, and skin. A clinical GVHD index was generated by summation of the five criteria scores (0–10). Survival was monitored daily. Animals with scores of 5 or more were considered moribund and were humanely killed.

Assessment of CMT-P815 (H-2^d) mastocytoma induction and assessment of mastocytoma death vs death from GVHD

B6D2F1/J recipients received 1×10^5 P815 (H-2^d) cells i.v. on day 0 of allogeneic HSCT (5×10^6 TCD BM cells and 5×10^5 T cells of C57BL/6 origin). Survival was monitored daily and the cause of death after HSCT was determined by necropsy by our veterinary pathologist Dr. H. T. Nguyen (Cornell University, New York, NY) as previously described. Briefly, death from leukemia was characterized by hepatosplenomegaly and the presence of mastocytoma cells in liver and spleen on microscopic examination, whereas death from GVHD was defined as the absence of hepatosplenomegaly and leukemic cells in liver and spleen, and the presence of clinical symptoms of GVHD as assessed by our clinical GVHD scoring system at the time of death.

Administration of IL-7

IL-7 was either given from days 0 to 13 or 21 to 27 i.p. at 10 μ g/day for immune reconstitution studies. PBS was injected into control mice at the same time points.

Thymic stromal cell isolation

Thymic stromal cells were isolated as described in Ref. 34. Briefly, thymic tissue from at least 10 mice/treatment group was digested in 0.125% (w/v) collagenase D (Roche Applied Sciences), then trypsin (Sigma-Aldrich) and 0.1% (w/v) DNase (Roche Applied Sciences) in RPMI 1640. Cells were incubated with anti-CD45 microbeads and depleted of CD45⁺ cells using an autoMACS (Miltenyi Biotec).

Semiquantitative RT-PCR

Total cellular RNA from whole BM and CD45⁺ thymic stromal cells was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies). cDNA was PCR-amplified for 35 cycles (94°C for 30 s; 56°C for 30 s; 72°C for 60 s) with PCR Master Mix (Promega).

HPRT: 5'-CACAGGACTAGAACACCTGC-3' and 5'-GCTGGTGAA AAGGACCTCT-3' TGFB1: 5'-CTACTGCTTCAGCTCCACAG-3' and 5'-TGCACTTCAGGAGCGCAC-3' and KGF: 5'-GCCTTGTCACGACCT GTTTC-3' and 5'-AGTTTCACTCGTAGCCGTTT-3'. IL-7: 5'-GCCT GTACATCATCTDAOTGC-3' and 5'-TGAACCACTAATCTCTTGA GGTTC-3'.

Enzymic digestion of IL-7^{-/-} thymi

IL-7^{-/-} mice, having a marked reduction in thymocyte development, contain a large proportion of CD45⁺ thymic stromal cells and hence each thymus was subjected to enzymic digestion in 0.125% (w/v) collagenase/dispase (Roche Applied Sciences) with 0.1% (w/v) DNase, to release most

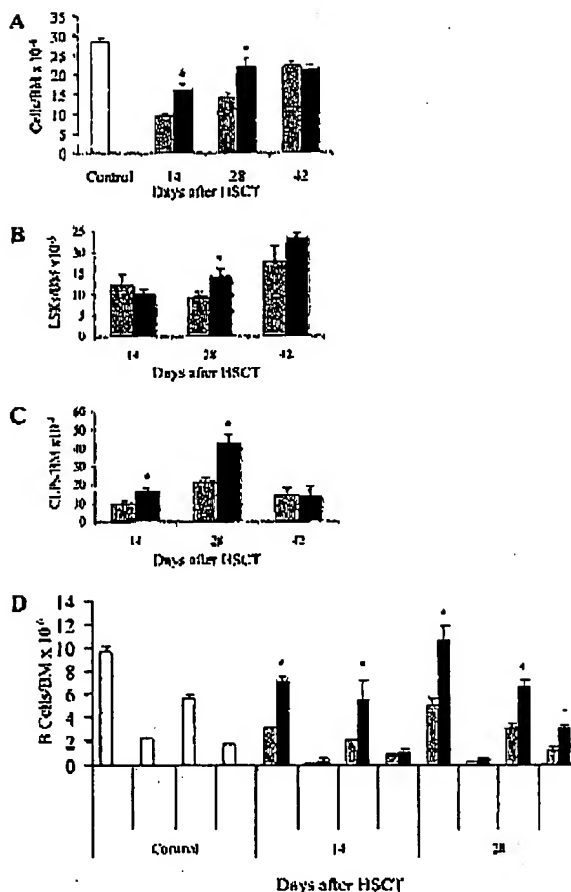


FIGURE 1. Castration enhances reconstitution of the BM in allogeneic HSCT recipients. Eight- to 12-wk-old male CBA mice were sham-cx or cx and transplanted with 5×10^6 B10.BR TCD BM cells. **A**, BM cellularity. **B** and **C**, Lineage mixture comprised CD3, CD8, CD4, CD45R, Gr1, CD11b, NK1.1, and TER119 donor-derived LSK (Ly9.1⁺Lin⁺c-kit⁺Sca-1⁺) (**B**) number and donor-derived CLP (Ly9.1⁺Lin⁺c-kit^{low}Sca-1^{low}TL-7R α ⁺) (**C**) number donor-derived B cell precursor number: pro-B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁻) (**D**); pre-B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁺), and immature B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁺). □, Age-matched, untreated controls; ▨, sham-cx mice; ■, cx mice. *, $p < 0.05$ and each group contained four to five animals.

of the stromal and lymphoid cells. This allowed for the accurate calculation of total cellularity. Stromal cells were identified as being CD45⁺.

Statistics

All values are expressed as mean \pm SEM. The Mantel-Cox log-rank test was used for survival data and all other statistical analysis was performed with the nonparametric, unpaired Mann-Whitney U test. A p value of <0.05 was considered statistically significant.

Results

Castration increases BM cellularity, HSC numbers, and B cell precursors

Male CBA mice were cx 1 day before allo-HSCT. There were significantly more cells in the BM ($16 \times 10^6 \pm 1.4 \times 10^6$) of cx mice, compared with the sham-cx controls ($9.5 \times 10^6 \pm 3.0 \times 10^5$) as early as 14 days after HSCT (Fig. 1A). These numbers remained elevated in

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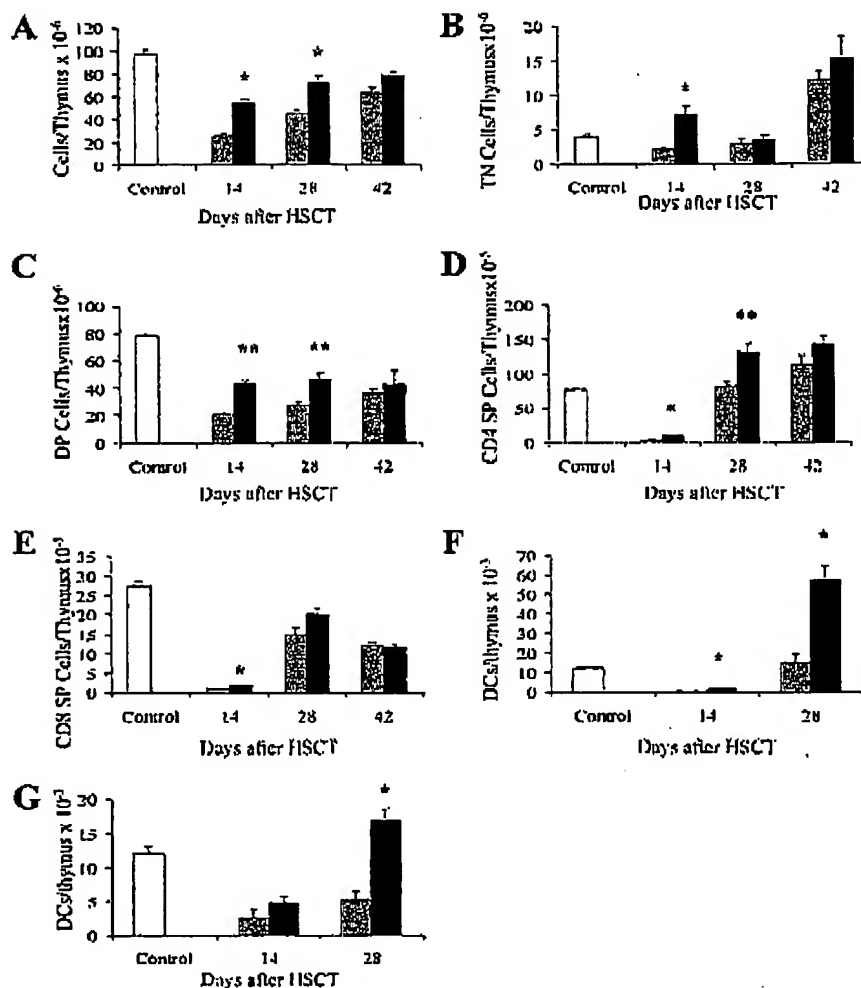


FIGURE 2. Thymic cellularity and donor-derived thymocyte and DC number are significantly increased following allo-HSCT and castration. Eight- to 12-wk-old male CBA mice were sham-cx or cx and transplanted with 5×10^6 B10.BR TCD BM cells. **A**, Thymic cellularity. **B**, Donor-derived TN cells (Ly9.1⁺CD3⁺CD4⁺CD8⁺). **C**, Donor-derived DP cells (Ly9.1⁺CD4⁺CD8⁺). **D**, Donor-derived mature CD4 SP cells (Ly9.1⁺CD3⁺CD4⁺CD8⁺). **E**, Donor-derived mature CD8 SP cells (Ly9.1⁺CD3⁺CD4⁺CD8⁺). **F**, Host-derived DCs (Ly9.1⁺CD11c⁺MHCII^{high}). **G**, Donor-derived DCs (Ly9.1⁺CD11c⁺MHCII^{high}). □, Age-matched, untreated controls; ▨, sham-cx mice; ■, cx mice. *, $p < 0.05$; **, ($p < 0.01$) and each group contained four to five animals.

cx mice at day 28 (BM: $22 \times 10^6 \pm 4.0 \times 10^6$ vs $14 \times 10^6 \pm 2.2 \times 10^6$). The cx mice had begun to approach pretransplant levels at this time point. By day 42, there was no longer a difference between cx and sham-cx mice with respect to BM cellularity.

Several studies have shown that scx steroids inhibit the proliferation and/or differentiation of early hemopoietic precursors (35–37). Therefore, the impact of castration on donor-derived LSK (Lineage[−]Sca-1⁺c-kit⁺) numbers in the allogeneic HSCT setting was investigated. The number of donor-derived LSK (Ly9.1⁺Lin[−]Sca-1⁺c-kit⁺) was not significantly different 14 days after allo-HSCT (Fig. 1B). By day 28, there were more Ly9.1⁺Lin[−]Sca-1⁺c-kit⁺ donor-derived LSKs in the BM of cx mice compared with the sham-cx controls. Donor-derived LSK numbers in both treatment groups were approaching pretransplant levels and there was no difference in donor-derived HSC number 42 days after allo-HSCT (Fig. 1B).

Donor-derived common lymphoid precursors, defined as Lineage[−]c-kit^{low}Sca-1^{low}IL-7R α ⁺, were also significantly increased in cx mice 14 and 28 days after allo-HSCT (Fig. 1C). This finding is in agreement with earlier studies that suggest that lymphoid progenitors are steroid sensitive (36, 37).

In our analysis of B cell recovery, three stages in B cell development were distinguished: pro-B cells (CD45R⁺CD43⁺

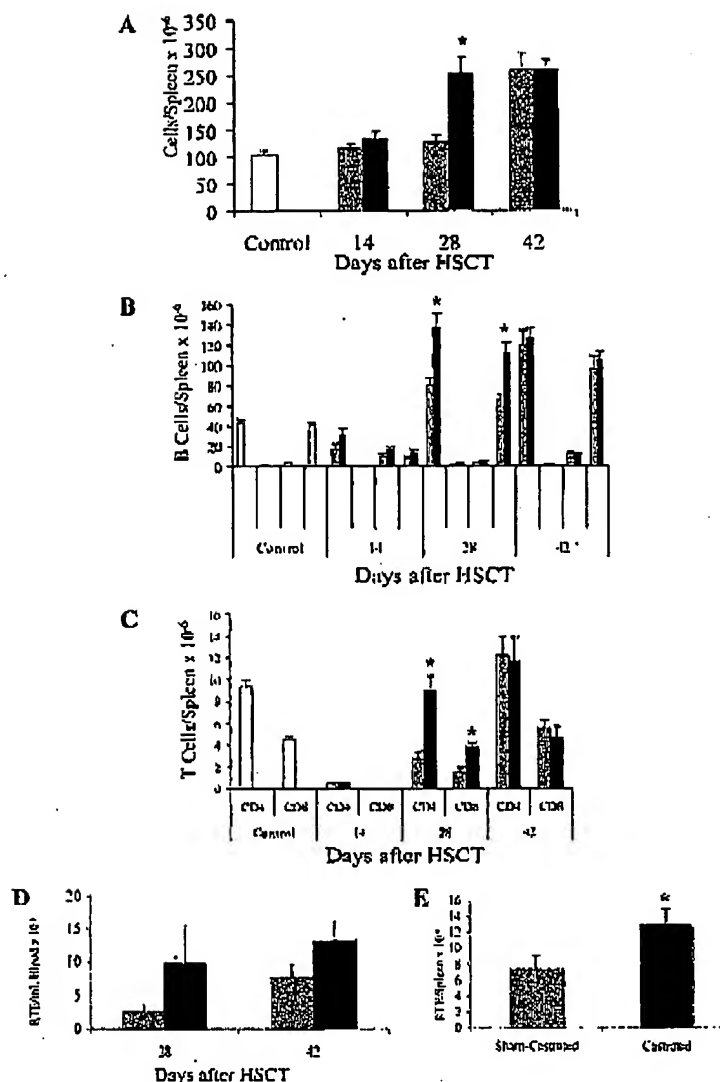
IgM[−]), pre-B cells (CD45R⁺CD43[−]IgM[−]), and immature B cells (CD45R⁺CD43[−]IgM⁺). As early as 14 days after allogeneic HSCT, pre-B cell numbers in the BM of cx mice had reached pretransplant levels ($5.5 \times 10^6 \pm 1.7 \times 10^6$) and were significantly higher than the sham-cx controls ($2.08 \times 10^6 \pm 5.0 \times 10^5$) (Fig. 1D). At day 28, again there were significantly more pre-B cells (sham-cx: $3.1 \times 10^6 \pm 3.7 \times 10^5$ c.f. cx: $6.6 \times 10^6 \pm 6.6 \times 10^5$) and immature B cells (sham-cx: $1.3 \times 10^6 \pm 2.6 \times 10^5$ c.f. cx: $3.0 \times 10^6 \pm 3.4 \times 10^5$) in the BM of cx mice (Fig. 1D).

Castration before allo-HSCT results in an increase in thymic cellularity as well as thymocyte and dendritic cell (DC) numbers

At the early time point of day 14, thymic cellularity is increased in cx mice ($55.4 \times 10^6 \pm 1.8 \times 10^6$) compared with sham-cx control ($25 \times 10^6 \pm 2.6 \times 10^6$) (Fig. 2A). These numbers remained significantly elevated in cx mice 28 days after HSCT ($72 \times 10^6 \pm 5.9 \times 10^6$ vs $45 \times 10^6 \pm 2.9 \times 10^6$). By day 42, there was no longer a significant difference between cx and sham-cx mice with respect to thymic cellularity.

Donor-derived thymocytes (Ly9.1⁺) were divided into developmental stages on the basis of expression of CD3, CD4, and CD8:

FIGURE 3. Castration enhances donor-derived peripheral T and B cell reconstitution in allogeneic HSCT recipients. **A–C.** Eight- to 12-wk-old male CBA mice were sham-cx or cx and transplanted with 5×10^6 B10.BR TCD BM cells. **A.** Splenic cellularity. **B.** Donor-derived B cell number based on the expression of CD45R/B220, IgM, and CD43. Pro-B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁺), pre-B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁺), and immature B cells (Ly9.1⁺B220/CD45R⁺CD43⁺IgM⁺). **C.** Donor CD4 T cells were Ly9.1⁺CD3⁺CD4⁺CD8⁺ and donor CD8 T cells were Ly9.1⁺CD3⁺CD4⁺CD8⁺. □, Age-matched, untreated controls; ▤, sham-cx mice; ■, cx mice. *, $p < 0.05$, **, $p < 0.01$ and each group contained four to five animals. **D and E.** Eight- to 12-wk-old male BALB/c mice were sham-cx or cx and transplanted with 5×10^6 TCD RAG2p-GFP Tg BM cells. **D.** Ly9.1⁺CD3⁺GFP⁺ RTEs in blood (per milliliter). **E.** Ly9.1⁺CD3⁺GFP⁺ splenic RTEs day 42 post allogeneic HSCT. *, $p < 0.05$ and each group contained five to seven animals.



triple negative (TN) (CD3⁺CD4⁺CD8⁺), double positive (DP) (CD4⁺CD8⁺), single-positive CD4 (SP CD4) (CD3⁺CD4⁺CD8⁺), and SP CD8 (SP CD8) (CD3⁺CD4⁺CD8⁺) (Fig. 2, B–E). There were no differences between sham-cx and cx mice when comparing the proportions of the different thymocyte subsets (data not shown). At day 14, the donor-derived thymocytes in both groups were predominantly TNs and DPs (data not shown). However, as early as 14 days after allo-HSCT, there were significantly more donor-derived TN, DP, SP CD4 and SP CD8 thymocytes in cx mice compared with sham-cx controls (Figs. 2, B–E). Twenty-eight days after HSCT, DP and CD4 SP cell numbers remain significantly elevated in the cx group. By day 42, all thymocyte subsets were equivalent in sham-cx and cx mice.

Host and donor-derived DCs are thought to play integral roles in the avoidance of self and graft rejection, respectively (38). Both host and donor-derived DCs in the thymus were significantly increased in the cx mice 14 and 28 days after allo-HSCT (Fig. 2, F and G).

Splenic cellularity is increased with more donor-derived peripheral T and B cells 28 days after castration and allo-HSCT

Splenic cellularity in the cx mice was significantly elevated above sham-cx spleen cell numbers 28 days after allo-HSCT ($253 \times 10^6 \pm 28.4 \times 10^6$ vs $126 \times 10^6 \pm 13.9 \times 10^6$) (Fig. 1C). The cx mice had begun to approach pretransplant cellularities by day 28. Again, this may have been because the recipients were young mice and they had active posttransplant lymphopoiesis which facilitated their recovery: the time required to generate normal cellularity in the primary and secondary lymphoid tissues in the sham-cx mice, however, was markedly delayed compared with cx recipients.

The increase in BM B cells and their precursors translated to a significant increase in the number of immature B cells in the spleens of cx mice, 28 days after HSCT (sham-cx: $64.9 \times 10^6 \pm 6.4 \times 10^6$ c.f. cx: $112.0 \times 10^6 \pm 10.0 \times 10^6$) (Fig. 3B). These

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ENHANCED IMMUNE RECONSTITUTION AFTER ALLOGENEIC HSCT

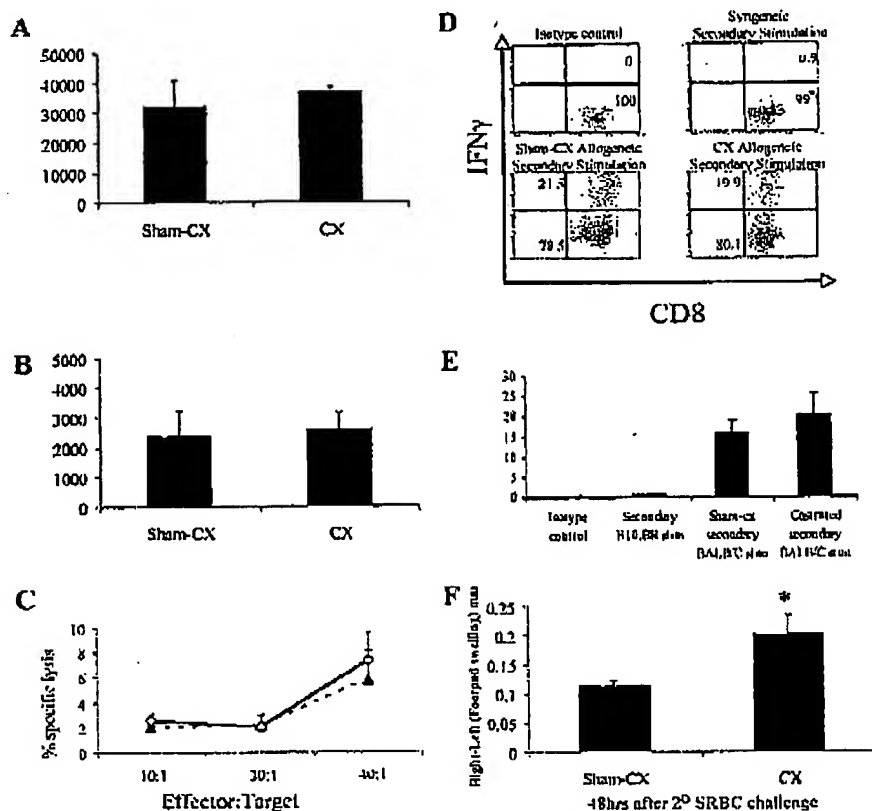


FIGURE 4. Castration does not alter the function of donor-derived T cells following allogeneic HSCT. Eight- to 12-wk-old male CBA mice were sham-cx or cx and transplanted with 5×10^6 B10.BR TCD BM cells. Forty-two days after transplantation, T cell functionality was assessed. **A**, Castration has no effect on the proliferative capability of T cells after allogeneic HSCT. Splenocytes were obtained from sham-cx ($n = 5$) and cx ($n = 5$) for proliferation assays and were cultured for 4 days with anti-CD3 and anti-CD28 (5 μ g plate-bound) and [3 H]thymidine was added during the final 18 h of culture. **B**, Alloreactive T cell proliferation. Splenic T cells (4×10^5 cells/well) were incubated with irradiated (20 Gy) BALB/c splenic stimulator cells (2×10^5 cells/well) in 96-well plates for 5 days and [3 H]thymidine was added during the final 20 h of culture. Each group contained five animals. **C**, Cytolytic activity of donor-derived T cells. Splenocytes were harvested from the transplanted sham-cx or cx mice (described in Fig. 1) and cultures (2×10^6 cells/well) for 5 days in 24-well plates with irradiated (20 Gy) BALB/C (third-party) splenic stimulator cells (1×10^6 cells/well). Cytotoxicity was determined against A20 (a BALB/c B cell lymphoma cell line) in a [51 Cr] release assay. **D** and **E**, Intracellular IFN- γ expression of alloreactive T cells. Splenic B6 T cells were harvested on day 42 from sham-cx or cx recipients as described above and incubated with irradiated (20 Gy) (BALB/c, third party) splenic stimulator cells in 24-well plates for 5 days. Cells were harvested, and restimulated with TCD, irradiated (20 Gy) (BALB/c or B10.BR [internal biological control]) splenic stimulator cells for 16 h. Brefeldin A (10 mg/ml) was added after the first hour of incubation. Intracellular IFN- γ expression in donor-derived CD3 $^+$ CD8 $^+$ cells was measured by flow cytometric analysis. Representative plots are shown in **D** and graphically represented as the percentage of donor-derived CD8 $^+$ T cells that express IFN- γ in **E**. **F**, Measurement of T cell functionality by DTH assay. DTH assay (see Materials and Methods) was performed at week 6 following allogeneic HSCT in sham-cx and cx mice, and the swelling was measured by subtracting left hind footpad swell from the right hind one.

results are in agreement with previous studies that suggest that castration enhances B cell production and export from the BM (31).

The increase in thymocyte numbers in cx mice translated to a significant increase in the number of donor-derived mature CD4 $^+$ and CD8 $^+$ T cells in the spleens of cx mice compared with the sham-cx controls at day 28 (Fig. 3C).

RTEs are a distinct population of naive, immature, peripheral T cells that have recently left the thymus (9, 39). In this study, we used mice that have a GFP transgene driven by the MLR 2 (RAG-2) promoter to identify RTEs. These mice, which have been used previously to identify RTEs (40), begin to express high levels of GFP at the CD4 $^+$ CD8 $^-$ double-negative stage of thymocyte development. GFP and RAG2 expression remain high throughout the CD4 $^+$ CD8 $^-$ DP stage of development and although RAG2

expression decreases with the SP transition, these cells remain positive for GFP through SP maturation and export. Peripheral T cells contain GFP $^{\text{high}}$, GFP $^{\text{low}}$, and GFP $^-$ populations, of which Boursalian et al. (40) have shown that the GFP $^{\text{high}}$ are the most recent emigrants.

In this study, we found an increase in donor-derived (Ly9.1 $^-$ GFP $^+$ CD3 $^+$) RTEs in the blood at days 28 and 42 (Fig. 3D) and spleen at day 42 (Fig. 3E) after HSCT in cx mice compared with sham-cx controls. The difference reached significance in the spleen at day 42 (Fig. 3E).

On a per cell basis, there is no significant functional difference between T cells from sham-cx and cx mice. To determine the functional potential of peripheral T cells in cx mice after allogeneic HSCT, a series of in vitro assays were performed. The proliferative capacity of the splenic T cells was tested in two

ways: anti-CD3/anti-CD28 cross-linking (Fig. 4A) and in a third-party MLR (using irradiated BALB/c splenocytes as stimulators) (Fig. 4B). There was no significant difference in the proliferative capacity of peripheral T cells when comparing sham-cx and cx mice in either of these settings. Forty-two days after allogeneic HSCT, splenocytes were cultured with irradiated BALB/c splenocytes (third party) for 5 days. Following 5 days of allogeneic stimulation, the vast majority of cells in culture were CD8⁺ T cells. Half these cells were used in a CTL (⁵¹Cr release) assay to determine the cytotoxicity of splenocytes from sham-cx and cx mice. Splenocytes were tested for their ability to kill ⁵¹Cr-loaded A20 (BALB/c B cell lymphoma tumor cell line) cells at different E:T ratios (Fig. 4C). There was no significant difference between sham-cx and cx mice with respect to cytotoxicity. The other half of the cells cultured for 5 days were restimulated overnight with either third-party (BALB/c) or syngeneic (B10.BR) irradiated splenocytes and brefeldin A to determine IFN- γ production. Fig. 4D shows IFN- γ production by donor-derived CD8⁺ splenic T cells following BALB/c primary stimulation and either BALB/c or B10.BR secondary stimulation (control). This is represented graphically in Fig. 4E. There was no significant difference in the proportion of IFN- γ -producing donor-derived CD8⁺ when comparing sham-cx and cx mice. To assess immune function in vivo, a DTH assay was used whereby 42 days after castration and allogeneic HSCT mice were sensitized with sheep RBCs. On day 46, they were challenged and 24 and 48 h later, footpad swelling was determined. The DTH response was enhanced 48 h after challenge when mice were cx at the time of allo-HSCT compared with sham-cx controls (Fig. 4F). Collectively, these functional assays demonstrate that the T cells in cx recipients are comparable on a per cell basis with T cells from sham-cx recipients and are capable of responding to novel Ags with intact proliferation, cytotoxicity, and cytokine production. However, the significantly more rapid T cell numerical reconstitution in cx recipients translates to an enhanced DTH response even at 42 days after transplant suggesting a persistence of the castration-mediated effects.

Castration prior to allogeneic HSCT does not exacerbate GVHD and maintains GVT activity

Both GVHD and GVT are mediated, primarily, by alloreactive donor-derived T cells, which are transferred with the allograft. Any treatment used to enhance immune reconstitution has the potential to exacerbate GVHD or, conversely, decrease GVT activity.

To assess the effects of castration on GVT activity, we injected the mastocytoma cell line P815 (H-2d) into B6D2F1/J recipients at the time of transplant. Animals that died during the experiment were autopsied and the cause of mortality (tumor vs GVHD) was determined. Mortality due to mastocytoma remained unchanged following castration (six of nine mice) when compared with sham-cx controls (five of eight mice). This suggests that castration does not diminish GVT response following HSCT (Fig. 5A).

To establish that castration does not have a stimulatory effect on alloreactive T cells of donor origin, GVHD was induced by the addition of allogeneic donor T cells to the allograft. Two models were used: a major mismatch model (C57BL/6J (H-2^b) into C3H/HeB6F1/J(H-2^b)) (Fig. 5B) and a minor mismatch model (B10.BR (H-2^b) into CBA/J (H-2^k)) (Fig. 5, C-E). Three different T cell doses were used in the minor mismatch model: 0.1×10^6 (Fig. 5C), 0.5×10^6 (Fig. 5D), and 1×10^6 (Fig. 5E). There was

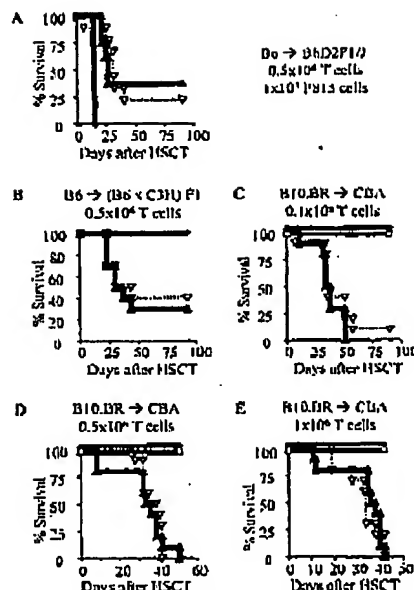


FIGURE 5. Castration administration does not aggravate GVHD or decrease GVT activity in allogeneic HSCT recipients. For A-E: Solid line and filled circle is a TCD-BM-only (no T cells) control group; solid line and outlined squares is a TCD-BM cx control group; solid line and filled triangle is the sham-cx treated group; and dotted line and outlined triangle is the cx-treated group. Control groups, $n = 4$; test groups, $n = 8-10$; survival is depicted as a Kaplan-Meier curve. A, GVHD: lethally irradiated, B6D2F1/J recipients received P815 (H-2d) cells (1×10^5). B, Major mismatch GVHD model: lethally irradiated (B6 \times C3H)/F1 recipients received transplants with B6 TCD BM cells (5×10^6) plus splenic T cells (0.5×10^6). C-E, Minor mismatch GVHD model: lethally irradiated CBA recipients received transplants with B10.BR TCD BM cells (5×10^6) and a range of T cell doses: C, 0.1×10^6 T cells; D, 0.5×10^6 T cells; E, 1×10^6 T cells.

no significant difference in morbidity or mortality due to GVHD when comparing cx and sham-cx mice (Fig. 5, B-E).

IL-7 and castration have an additive effect following allogeneic HSCT

We and others have previously shown that IL-7 treatment can increase the number of T and B cells in otherwise untreated animals and can also enhance lymphoid recovery following severe immunodepletion (32, 41-43). IL-7 is known to increase T cell numbers through increased thymic activity as well as peripheral expansion (44). We therefore assessed the effects of IL-7 administration in combination with castration following allogeneic HSCT. Fourteen days after treatment there were significantly more cells in the thymus of cx mice and those given the combined treatment (castration and IL-7 administration). At this early time point, there was no difference seen between the PBS-treated, sham-cx controls and the IL-7-treated, sham-cx mice. There was also no significant difference seen between the cx group and those receiving the combined treatment, suggesting that it is only the effects of castration acting 14 days after allo-HSCT, IL-7 treatment, and castration (Fig. 6A). At a later time point, day 28, the cellularity of the thymus in both the castration alone group and the IL-7 alone group is significantly higher than the control

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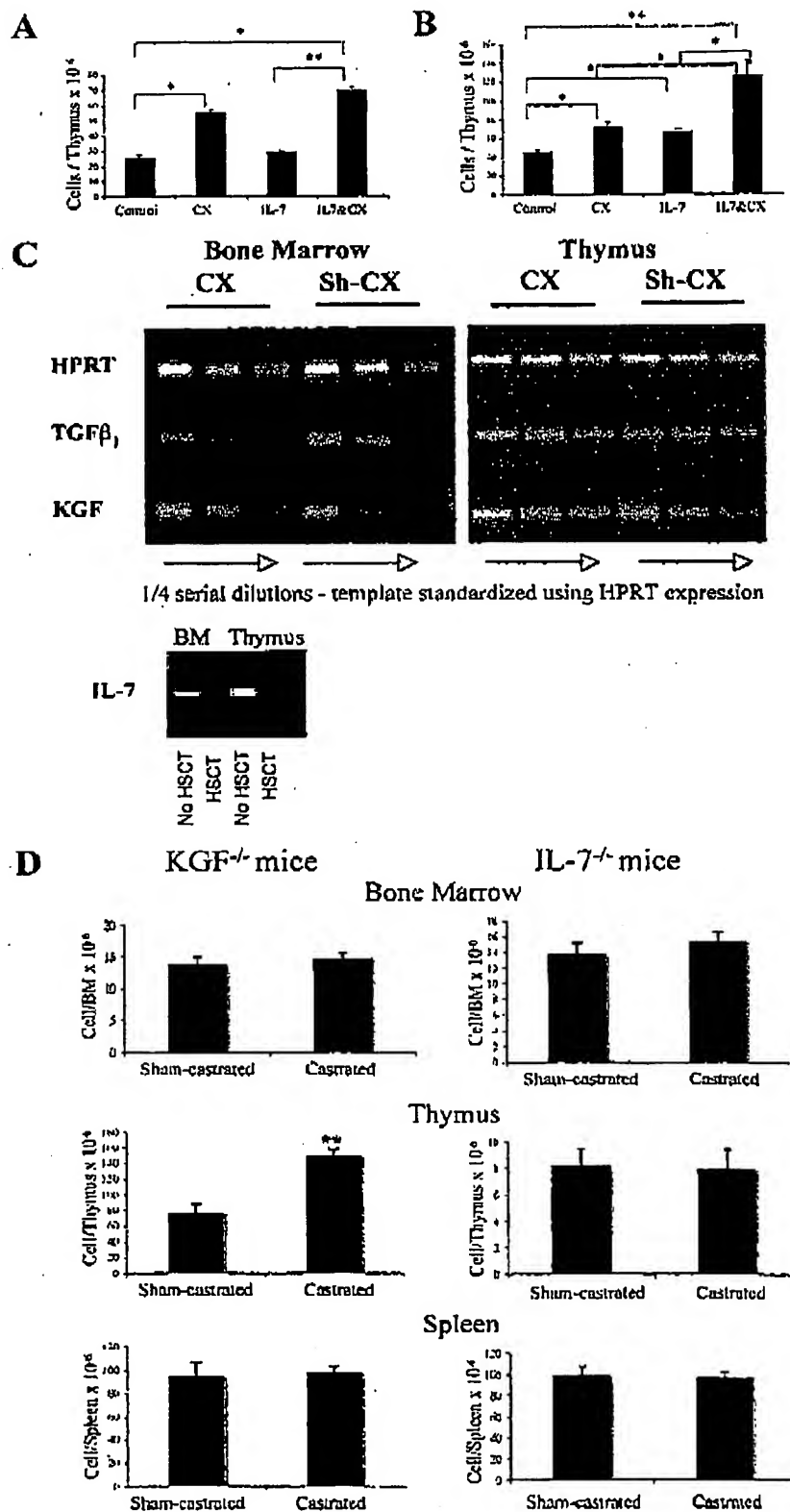


FIGURE 6. Casuarion and IL-7 treatment have an additive effect in the thymus following allogeneic HSCT. Eight- to 12-wk-old male CBA mice were sham-cx or cx and transplanted with 5×10^6 B10.BR TCD BM cells. Recipient organs were harvested on day 14 (A) received in addition 10 μ g/day IL-7 or PBS (control) by i.p. injection from days 0 to 13. Recipients killed on day 28 (B) received 10 μ g/day IL-7 or PBS from days 21 to 28. Thymic cellularity was calculated from total cell counts. *, $p < 0.05$, represents a significant increase in cell number in the cx group compared with the sham-cx control. Control indicates, sham-cx, PBS injected; CX: cx and PBS injected; IL-7: sham-cx and IL-7 injected; and IL-7 and CX: cx and IL-7 injected. C, Semiquantitative RT-PCR was performed on whole BM and CD45⁺ thymic stroma (at least 10 mice/treatment group) 14 days after allogeneic HSCT and casuarion. After HPRT equilibration templates from cx and sham-cx mice were compared for the expression of TGFβ₁ and KGF. These results have been confirmed using BM and thymic stromal template from a second experiment. IL-7 remained undetectable in the BM and thymus for up to 28 days after HSCT. D, KGF^{-/-} and IL-7^{-/-} mice ($n = 6-8$) were sham-cx or cx and 14 days later thymic, BM, and splenic cellularity were analyzed.

group. In addition, the combination of IL-7 treatment and castration had an additive effect on thymic cellularity at day 28 after allogeneic HSCT (Fig. 6B).

Semiquantitative RT-PCR for IL-7, TGF- β , and KGF reveals an increase in KGF and a decrease in TGF- β following allogeneic HSCT and castration

RT-PCR analysis of whole BM cells revealed undetectable levels of IL-7 transcript in both sham-cx and cx mice as late as 42 days after allogeneic HSCT (Fig. 6C). When template from control, untransplanted mice were used IL-7 was detected (Fig. 6C). TGF β , and KGF are known to be key mediators of hematopoiesis. Using 4-fold serial dilutions of template from at least 10 mice per treatment group, templates were hypoxanthine phosphoribosyltransferase (HPRT) equilibrated and there appeared to be a decrease in TGF β , and an increase in KGF 14 days after castration and allo-HSCT (Fig. 6C). These results are reproducible with samples from within the same experiment and samples from a second independent experiment. CD45⁺ thymic stromal cell TGF β , and KGF levels were also tested. There was no visible difference in either growth factor when comparing template from sham-cx and cx mice.

Changes that occur following castration were seen in KGF^{-/-} mice but not IL-7^{-/-} mice

To further study the possible mechanisms behind the enhanced immune reconstitution following castration, KGF^{-/-} and IL-7^{-/-} mice (4–6 mo old $n = 6–8$) were cx and 14 days later, thymus, spleen, and BM were analyzed (Fig. 6D). TGF β ^{-/-} mice could not be analyzed because they die prepubertally (45). Thymic cellularity was significantly ($p < 0.01$) increased when comparing sham-cx and cx KGF^{-/-} mice. Although no differences were seen in the total cellularity of the BM and spleen at this early time point, changes were seen in the B cell compartment of the BM, as seen previously in wild-type mice (31) (data not shown). Due to the fact that a large proportion of cells in the thymi of IL-7^{-/-} mice are CD45⁺ stromal cells, enzymic digestion was used to obtain a single-cell suspension when using these mice. By doing this, many more cells are released into suspension which accounts for the slightly larger thymic cellularity seen in this experiment compared with previous literature (46). No differences were seen in the thymi, spleen, or BM of IL-7^{-/-} mice when comparing cx mice and sham-cx controls (Fig. 6D). Taking into account the variability within these experiments, it would appear that KGF is not obligatory for castration-induced immune recovery, while IL-7 may play a role.

Discussion

Recipients of an allogeneic HSCT experience a prolonged period of immune deficiency, which is often associated with life-threatening infections. With increasing age of the recipient, the risk of infection increases as does the time it takes for full immunological reconstitution. The period of immunodeficiency following HSCT can be >1 year and recent long-term studies demonstrated a decrease in TCR excision circle⁺CD4⁺ T cells in older HSCT patients compared with their donors (47, 48). This suggests that thymic damage and the subsequent decline in T cell production may be more prolonged than once thought. The majority of post-HSCT infections are associated with a lack of CD4⁺ peripheral T cells (49).

It is widely accepted that there is an association between the clinical outcomes of HSCT and the number of transplanted cells (50–53). Transplantation of an insufficient number of progenitor cells may lead to delayed and reduced immune reconstitu-

tion and an increase in transplant associated morbidity and mortality (50–53). In this study, we have shown that there are significantly more donor-derived lineage⁺Sca-1⁺c-kit⁺ LSKs and CLPs when castration is performed before allo-HSCT. This finding may allow for a decrease in the number of cells required for a viable transplant.

Thymic production of naive T cells of a diverse TCR repertoire is essential for the establishment of normal T cell function following allo-HSCT (54–56). In an earlier study, we presented preliminary data that sex steroid ablation induced by agonist LHRH increased overall thymocyte number (27), however, we did not examine the nature or function of these T cells, nor the impact on GVHD and GVT. We also did not examine any effects on BM recovery in allogeneic. Therefore, in the present series of experiments, castration of mice before allo-HSCT has been shown to reverse thymic damage caused by the conditioning regime and to enhance thymic reconstitution following allo-HSCT. The increased thymopoiesis was reflected across all thymocyte subsets. At the earliest time point, the donor-derived thymocytes were predominately TNs and DPs. The more mature donor-derived SP cells followed at days 28 and 42. At the later time points, in both the sham-cx and cx groups, the proportion of donor-derived thymocytes was equivalent to that of an untreated thymus suggesting normal thymopoiesis and a lack of evidence for pathological T cells in the postcastration setting. The changes observed in the thymus translated to an increase in donor-derived peripheral T cells and we have shown that this increase was at least in part due to an increase in thymic export.

T cell-mediated immune responses are known to be diminished for an extended period following allo-HSCT. In this study, we demonstrated that when tested, *in vitro* T cell function did not differ on a per cell basis when comparing cx and sham-cx mice following allo-HSCT. Using DTH as a measure of *in vivo* T cell function, castration before allo-HSCT lead to a stronger DTH (T cell-mediated) response than that seen in control animals, suggesting that sex steroid ablation may enhance T cell function following HSCT in a persistent manner.

Although B cell reconstitution following HSCT is comparatively fast, functional deficiencies in these cells are present for extended periods after transplantation (57). In this study, we have shown that castration before allo-HSCT results in an increase in donor-derived B cell lymphopoiesis and subsequent increase in peripheral B cell numbers (also donor derived). Hence, as for T cells, sex steroid ablation leads to accelerated normalization of B cell numbers which may lead to an increase in B cell function.

DCs are the key mediators of negative selection in the thymus (58, 59) and in a transplant setting have been implicated in inducing graft acceptance by presenting alloantigens in the thymus after transplantation, deleting newly arising donor-specific T cells. For example, donor-derived cells in the thymi of MHC class I-mismatched recipients mediate deletion of donor-reactive cells (60). It has also been shown that thymus-derived DCs injected *i.v.* traffic to the host thymus (61), however, whether this occurs physiologically is unclear. Also, intrathymic injection of host cells pulsed with alloantigen, donor cells, or donor-soluble peptides increases graft acceptance (62–65). In the current study, castration significantly increased the number of host and donor-derived DCs in the thymus following allogeneic HSCT. It is therefore possible that castration, used in conjunction with hematopoietic stem cell and solid organ transplantation, may increase graft acceptance. This is particularly relevant in the clinic where the vast majority of transplants are performed on older adults in whom thymic function is

minimal and hence HSC uptake for chimera formation greatly retarded.

Although they very likely act via intermediate cell types, both estrogen and testosterone can directly affect the differentiation and proliferation of HSCs (35–37). Estrogen directly inhibits the proliferation and differentiation of HSCs as well as some lymphoid precursor subsets (36, 37). HSCs express functional estrogen receptors (ERs) and estrogen administration decreases the number of Lin[−]c-kit⁺Sca-1⁺ HSCs (35, 37). Thurmond et al. (35) suggest that the transition between c-kit⁺Sca-1⁺ precursors and the more mature subsets (c-kit⁺Sca-1[−] and c-kit[−]Sca-1[−]) is blocked when ER α is present in the hemopoietic cells of the BM (35). ERs are also present on BM stromal cells (66, 67), suggesting that estrogen may also have an effect on the production of growth factors by the stroma, which in turn affects HSC proliferation and/or differentiation.

RT-PCR of the BM in the present study provided evidence for an increase in KGF and a decrease in TGF- β —both potential molecules involved in the castration-induced enhanced BM function. Use of KGF^{−/−} and IL-7^{−/−} mice demonstrated that both of these may be required, yet there is also a wide body of evidence implicating TGF- β .

Batard et al. (68) have demonstrated that physiological concentrations of TGF- β inhibit the proliferation and differentiation of HSCs in vitro. As a corollary, disruption of TGF- β signaling in HSCs (via the transient expression of a mutant type II receptor) enhances survival and proliferation of these cells (69). These findings are entirely consistent with the possibility that the increased number of HSCs seen 28 days after allogeneic HSCT and castration may in fact be due to a decrease in the production of TGF- β by BM stromal cells, as indicated from the RT-PCR analysis.

Several studies have shown that sex steroid ablation, be it by surgical or chemical castration, of male mice increases both BM and splenic B cell numbers (31, 70–72). Olsen et al. (73) have demonstrated that androgens enhance the production of TGF- β by stromal cells within the BM, which in turn suppresses B cell development (73). In addition, neutralization of TGF- β in vitro reverses B cell suppression by dihydrotestosterone (73). It is therefore possible that in our setting of sex steroid ablation, the opposite is occurring. A decrease in androgens may lead to suppression of TGF- β production, enhancing B lymphopoiesis.

Sex steroid ablation reverses age-related thymic atrophy (3–7). What remains to be fully understood is the mechanism by which this occurs. Using transfer experiments with wild-type and testicular feminization (tfm) mice, which have a point mutation in the androgen receptor, Olsen et al. (74) have shown that it is the presence of a functional androgen receptor on the thymic epithelium but not on the thymocytes that is essential for age-related thymic involution and the subsequent regeneration via sex steroid ablation.

Although the molecular mechanisms for thymic involution and age-related B cell defects (and their subsequent reversal) remain unclear there are several potential candidates. Thymic IL-7 levels decline with age (2, 75, 76), but it is unclear whether this is due to a decrease in the number of cells that produce IL-7 or a decrease in the ability of the existing cells to produce the cytokine. IL-7 treatment of old mice can reverse age-related increases in thymic apoptosis and enhance thymopoiesis (77). Stem cell factor and M-CSF mRNA expression is also decreased in the mouse thymus with age (75). Sempowski et al. (78) have monitored mRNA steady-state levels in aging humans and shown a significant increase in leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA.

The above studies suggest that it is unlikely that castration affects a single growth factor and it is more likely that the response is multifactorial. Our experiments with castration of IL-7^{−/−} mice (Fig. 6B) suggest that increased production of IL-7 is an important component of the castration effect. However, we observed an additive effect on thymic cellularity when recipients were treated with both high-dose IL-7 and castration, which would suggest that castration provides more thymopoietic effects than increased IL-7 levels alone. The current study has clearly demonstrated that sex steroid blockade has a profound positive effect on immune reconstitution following myeloablation and allo-HSCT. HSC and B and T cell progenitor recovery was enhanced leading to increased T and B cell production in the primary lymphoid tissues and a subsequent increase in donor-derived peripheral lymphocytes. Furthermore, in vivo T cell function, as tested by DTH, is augmented. This provides an important platform for increasing the efficiency of engraftment and posttransplant strategies that depend on an intact hemopoietic system.

When developing immune-enhancing treatments in the setting of allo-HSCT it is essential to also address the unique dichotomy of GVHD and the GVT response. In this study, we have established that while sex steroid ablation enhanced in vivo T cell function, GVT activity was maintained and, importantly, GVHD was not exacerbated.

Collectively, these results suggest that transient sex steroid ablation (using, for example, LHRH agonists or antagonists) could be developed as a prophylactic therapy to enhance posttransplant immune reconstitution in allogeneic HSCT.

Disclosures

Richard Boyd, Ann Chidgey, Jayne Sutherland and Gabrielle Goldberg are named as inventors on patents pertaining to this work.

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